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13. ABSTRACT (Maximum 200 Words)

Currently, there is no curative treatment for recurrent prostate cancer (PC). Although, radiation therapy (RT) has been successful in the treatment of primary PC, it is not used to treat locally recurrent PC because of the high morbidity of pelvic reirradiation. Hyperthermia has undergone extensive study as a treatment for BPH and its safety is very well established. Our long-term goal is to develop hyperthermia as a tumoricidal therapy for recurrent PC. We propose a strategy for *in situ tumor vaccination* where PC cells release tumor antigens following local treatment with hyperthermia, while cytokine-stimulated dendritic cells (DCs) harvest these antigens to mount an effective tumor-specific immunity in vivo.

Our proposal has the following specific aims-
Specific Aim I. To determine whether DCs are activated following antigen uptake from PC cells treated with Hyperthermia.
Specific Aim II. To determine whether DC-stimulating cytokines (GM-CSF, Flt3L and CD40L) following local hyperthermia of primary tumor induce specific immunity and improve local and distant tumor regression.

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A. INTRODUCTION

Prostate cancer is the most common malignancy in American men and the second most common cause of death. Although, radiation therapy has been offered for the organ-confined disease, it has been failed to eradicate the local disease. Currently there is no curative treatment for recurrent PC. Microwave hyperthermia has undergone extensive studies as a treatment for BPH and its safety is very well established. Our long-term goal is to develop hyperthermia as a tumoricidal therapy for recurrent PC. We *hypothesize* that prostate tumor cells that have been treated with hyperthermia (HT) could potentially serve as a source of tumor antigens in vivo, where dying tumor cells would release tumor antigens slowly over time. In addition, by introducing heat shock proteins (hsp), this local treatment regimen would provide a "danger" signal to the innate immunity and also release hsps that chaperone antigenic peptides from tumors and present them to antigen-presenting cells (APCs), such as dendritic cells (DC). We further hypothesized that following local therapy of PC, immunotherapy with DC activating cytokines such as GMCSF would provide an environment for tumor antigen uptake, processing and presentation to the treated tumor. Thus we present a strategy for in situ tumor vaccination where PC cells release tumor antigens following local treatment with hyperthermia, while cytokine- stimulated DCs harvest these antigens to mount an effective tumor-specific immunity in vivo. Our proposal has the following specific aims-

Specific Aim I. To determine whether DCs are activated following antigen uptake from PC cells treated with Hyperthermia.

Specific Aim II. To determine whether DC-stimulating cytokines (GM-CSF, Flt3L and CD40L) following local hyperthermia of primary tumor induce specific immunity and improve local and distant tumor regression.

B. BODY

B.1. Hyperthermia induces apoptosis of tumor cells *in vivo*. RM-1 cells were incubated in 43.7°C for 1 hour. Twenty-four hours after heat treatment, control cells (incubated in 37°C) and heat-treated cells were stained with Hoechst stain and apoptotic cells were counted. There were significantly more apoptotic cells after hyperthermia treatment (Figure 1a,b). In order to determine whether hyperthermia treatment (HT) of tumor in vivo also induces apoptosis, RM-1 tumors were grown on the dorsum of foot of C57Bl/6 mice. Two to three weeks old palpable RM-1 tumors were treated with HT (43.7°C for 1 hr). At various time points, the tumors were excised and tumor cells were harvested for FACS analysis after PI/Annexin V-FITC staining. One day after HT, there was significant increase in apoptotic cells in HT-treated RM1 tumors (90±5%), compared to untreated controls (<1%) (Fig.2).

Mouse PC cells, RM-1, apoptose after hyperthermia treatment (43.7°C for 1 hour)

Hoescht staining of RM-1 PC cells 24 hours after heat treatment

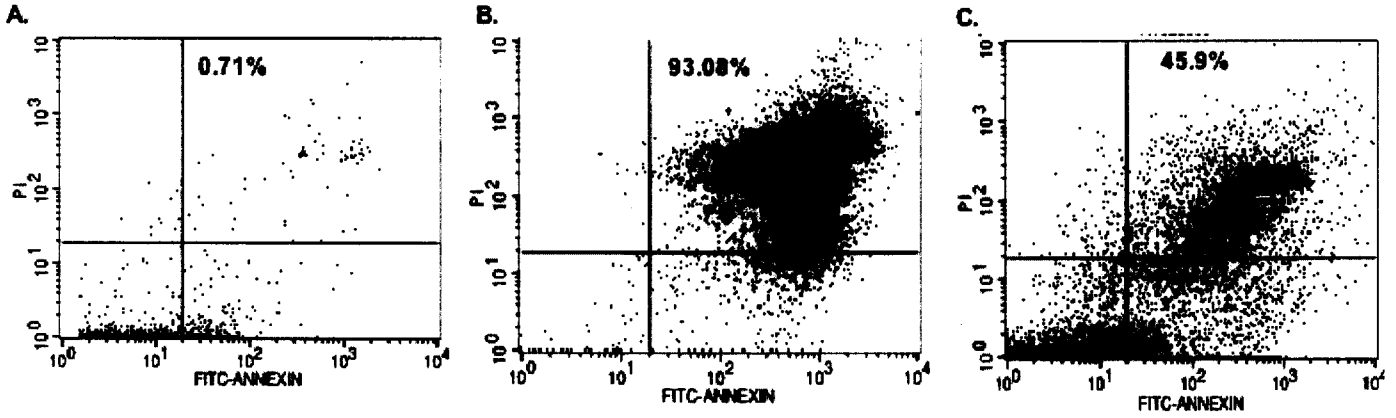


Fig 2. Hyperthermia induces apoptosis in HT-treated RM-1 cells in vivo. **A.** Untreated tumor; **B-C.** RM-1 tumor cells, 24 hours (B) and 48 hours (C) after HT (43.7°C for 1 hour). Note the presence of FITC-Annexin+ve apoptotic tumor cells in HT-treated RM-1 cells.

B.2. DCs are activated following antigen uptake from PC cells treated with hyperthermia. Autologous DCs were prepared from bone marrow derived cells after in vitro culture with GM CSF and IL-4 according to published methods. Bone-marrow derived, immature DCs have efficient antigen uptake capability. Previously we have shown that immature BM DCs can engulf flurochrome labeled hepatocellular carcinoma cells (HCC) and after engulfment efficient maturation signals are provided to them and mature DCs induce the expression of cell surface costimulatory molecules. Here in this study we examined whether immature BM DC can become mature after engulfment of heat treated RM1 cells and express costimulatory signals for T-cell activation. So, we treated the RM1 cells with 43.7°C for 1 hr., in another plate we treated the RM1 cells with 30 Gy radiation and one plate we left as untreated. One day after treatment we co- incubate separately all of them with resting immature BM DC. FACS analysis showed that when BMDC co incubated with untreated RM1 cell there is only 10% increase in CD80 expression but when they are incubated with heat treated RM1 cells, there is about 31% increase in CD80 expression and in case of radiated RM1 cells there is 36% increase in CD80 expression. (Fig. 2 A-C).

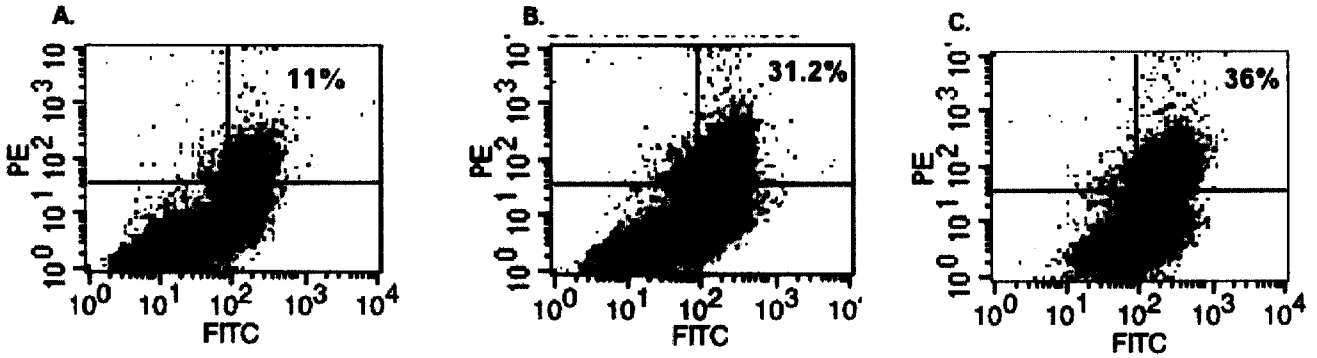


Fig 3. Induction of CD80 cell surface expression in dendritic cells (DC) following coculture with RM-1 tumor cells. A Untreated; B. HT-treated (43.7°C for 1 hour); and C. Irradiated (30 Gy) RM-1 cells. Tumor cells after various treatments were co-cultured with bone-marrow-derived autologous, DCs for 24 hours. DCs were then stained with FITC-labeled anti-mouse CD80 and PE-labeled, anti-mouse CD11c.

B.3. Hyperthemia retards tumor growth in C57Bl/6 mice. Mouse prostate cancer cells, RM-1 ($1x10^5$ cells), were injected into the dorsal side of the foot of C57Bl/6 mice. Two to three weeks after tumor cell inoculation, palpable tumors were treated with hyperthermia (43.7°C) in two treatments 3 to 5 days apart. Compared to untreated controls (n=6), hyperthermia (n=10) retarded the tumor growth significantly ($p<0.001$)(Fig. 4).

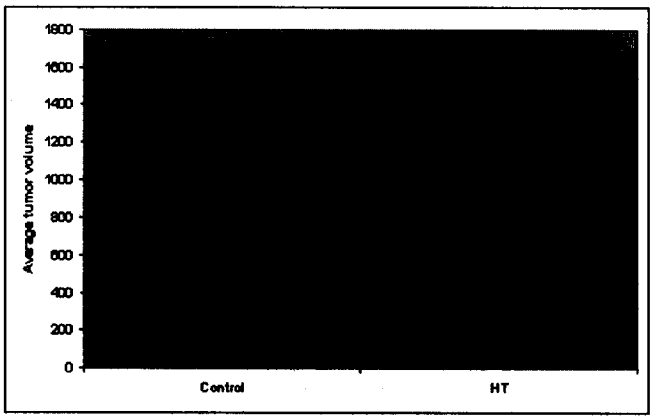


Fig. 4. Hyperthemia (43.7° C for 1 hr, 2 doses seperated by 5 days) retards tumor growth compared to control

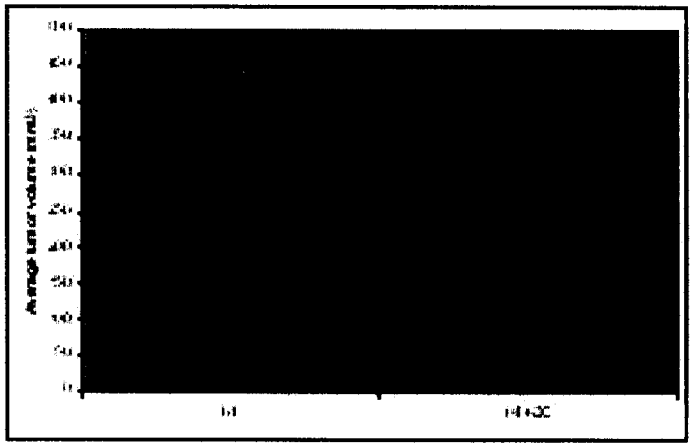
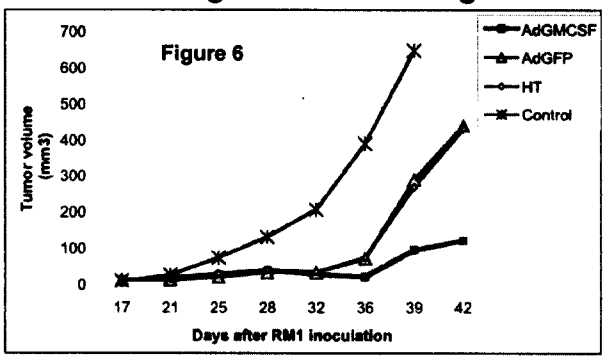


Fig. 5. Tumor growth retardation by intratumoral DC (1×10^6) injection following HT (43.7°C for 1hr)

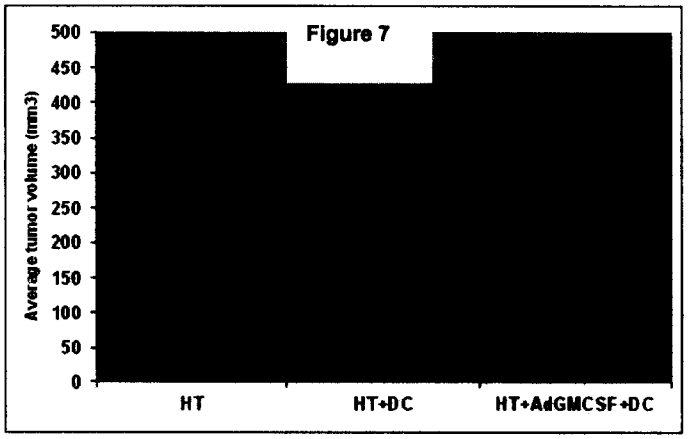
B.4. Intratumoral DC injection following hyperthermia showed significant tumor growth delay. It was observed from our *in vitro* data that DC matures after engulfing apoptotic and necrotic cells and express costimulatory signal for inducing T cell activation and we have also demonstrated that HT induces apoptosis in tumor cells both *in vivo* and *in vitro*. In order to examine whether intratumoral DC inoculation would enable DCs to engulf tumor antigens from HT-treated tumor cells and present it to T cells for induction of tumor-specific cell-mediated immunity, we injected BM-derived, autologous, DCs intratumorally. Three-week old RM1 (approx. 100mg) tumors were subjected to HT (43.7°C for 1 hr) in two doses, separated by 3 to 5days. Following second HT treatment, animals received either PBS or DCs (10^6 per injection) intratumorally every three days for three injections. Intratumoral DC inoculation (n=16) significantly (p=0.007) augmented the tumoricidal effects of HT (n=10) (Fig.5). We also examined the T helper cell activity and cytotoxic lymphocyte activity induced after DC inoculation. Results are shown in section B.7. and it demonstrates an induction of tumor-specific immune response. Therefore, intratumoral DC injection induced a protective immunity against HT-treated tumor cells, thereby augmenting the tumor control rates after HT.

B.5. **Systemic administration of adeno-GM-CSF induces DC infiltration in heat-treated tumor and causes significant tumor growth delay.** Since GM-CSF increases the amount of circulating DCs in peripheral blood, we examined whether systemic administration of a recombinant adenovirus expressing murine GM-CSF can increase infiltration of DCs in HT-treated tumors and generate a T cell-mediated immune response. Three-week old RM1 (approx. 100mg) tumors, were subjected to HT (43.7°C for 1 hr) x 2 doses separated by 5days. Following HT treatment, animals received either Adeno murine GM-CSF (8×10^9 particles) i. v. or adenoLacZ or adenoGFP. Neither systemic injections of control adenoviruses (LacZ and GFP) were responsible for RM1 tumor volume reduction in HT-treated animals (p=0.67). All animals were sacrificed when the HT+Ad-GFP-treated cohorts had tumor size greater than 600 mm^3 tumor (42-50 days after RM-1 inoculation). AdGM-CSF (n=13) significantly retarded tumor growth (p=0.001) in HT-treated RM-1 tumors (Fig 6). Cell suspensions from excised tumors were stained with PE-conjugated antimouse CD11c monoclonal antibody and analyzed in FACS. The result demonstrated infiltration of DCs and CD4+ve and CD8+ve T cells.



B.6. **Systemic administration of Adeno-GM-CSF further augments the effect of intratumoral DC inoculation.** We also examined whether GM-CSF can augment the effects of intratumoral DC by

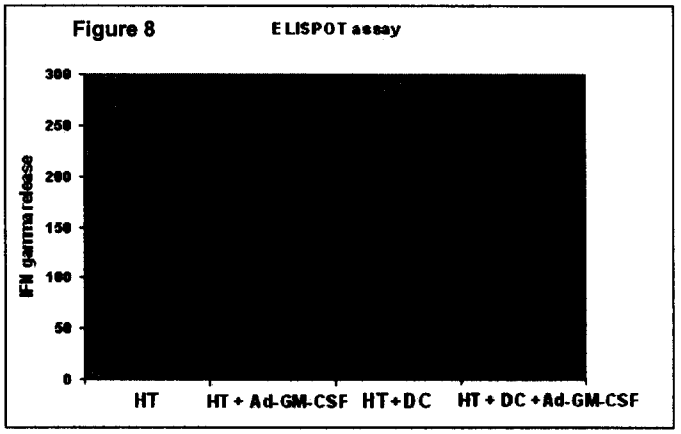
stimulating its matiuration. Three-week old RM1 (approx. 100mg) tumors were subjected to HT (43.7°C for 1 hr) in two doses separated by 5days. Following second HT treatment, animals received either PBS or DCs (10^6 per injection) intratumorally every three days for three injections. After 1st hyperthermia treatment Adeno-mGMCSF (8 x10^9) was administered i. v. in separate cohorts. Control animals received adenoLacZ or adenoGFP. It was observed that HT alone (n=10) was responsible for tumor growth delay when compared to untreated controls (Fig.4, 5). Intratumoral DC inoculation (n=16) augmented the tumoricidal effects of HT, significantly (p=0.007). Also it was observed that compared to only hyperthermia treatment GMCSF treated animals showed significant tumor growth delay (Fig.7, 8). Systemic injection of control adenoviruses (LacZ and GFP) did not alter tumor control of HT-treated animals (p=0.67). Administration of AdGMCSF (n=12) further enhanced the tumoricidal effects of HT+DC (p<0.001) (Fig.7).



B.7. Induction of systemic immune response following DC + Ad-GM-CSF treatment.

B.7.1. Induction of cytokines and T helper immunity.

ELISPOT assay for IFNγ. Briefly, 96-well nitrocellulose-based microtiter plates were coated overnight at room temperature with anti-IFNγ MAbs, diluted in PBS, per well. After the plate was washed with PBS, all wells were blocked with PBS+1%BSA for 2 h at 37°C. The splenocytes from immunized and control mice were then added to the wells (1 x 10^6 to per well) in RPMI 1640-10% FCS (50 µl/well)and RM1 cells (50 µl/well) were added to the wells containing splenocytes (1 x 10^5 per wells) and incubated for 30 hr at 37°C in 5% CO_2. After the wells were washed in PBS followed by PBS-Tween, biotinylated anti IFNγ MAbs was added, diluted in PBS-Tween and then incubated for an additional 2 hr at 37°C. Plates were washed in PBS-Tween, and streptavidin-HRP conjugate was added to each well and incubated (1 hr and 30 min). Spots representing a single cytokine secreted by individual cells were developed by using the peroxidase substrate 3-amino-9-ethylcarbazole and analyzed by ELISPOT analyzer. Mean numbers of spot-forming cells (SFC) were calculated from triplicate assays. The results are representative means (n = 3-8) ± standard deviations from separate experiments of treatment cohorts (Fig 8): AdGMCSF, DC and the combination of AdGMCSF + DC in the presence of localized tumor hyperthermia (43.7C for 1hr x 2 q 5dys). The addition of GM-CSF to HT+DC significantly ($p \geq 0.001$) augmented the IFNγ production of splenic lymphocytes, when compared to RM1 stimulated lymphocytes from either HT + GMCSF or HT + intratumoral DC cohorts. Interestingly, the presence of either HT + GMCSF or HT + intratumoral DC were not significantly different from each other, but they were significantly greater than the affects of HT alone on IFNγ production. Thus DC-based therapies induce RM-1-specific T helper lymphocytes.



B.7.2. Induction of CTL response. For CTL assays (LDH release assay), splenocytes from immunized mice were suspended in complete RPMI-1640 containing 10% FBS and the cells were stimulated with RM1 cells; the cells were then analyzed for cytotoxic activity 5 days after in vitro stimulation. LDH-release assay was performed in a 96-well round bottom plate using RM1 cells as

target cell. CTL assays were performed at lymphocyte effector: target (E:T) ratios of 10:1. The results were expressed according to the formula:

$$\% \text{ specific analysis} = \frac{\text{(experimental release- spontaneous release)}}{\text{(maximum release- spontaneous release)}}$$

From the LDH release assay we observed that DC injection induced tumor-specific CTL activity (LDH release 17-20% vs 5% in control). AdGMCSF alone with HT showed CTL activity (aprrox. 19% LDH release) comparable to HT+DC. The addition of Ad-GM-CSF augmented the CTL response to 36.3% (range, 30-44%) when given in combination with HT+DC, indicating amplification of tumor-specific immune response by adjuvant GM-CSF.

B.8. Future Experiments.

B.8.1. Amplification of DC maturation by CD40L and ATRA. In order to amplify the anti-tumor immune response, we would examine whether providing maturation signals (CD 40L and all-trans-retinoic acid) to intratumoral DCs would prolong and intensify the anti-tumor immune response. A recombinant adenovirus expressing CD40L has been constructed and experiments are underway to evaluate these hypotheses.

B.8.2. Prolongation of antitumor immunity by administration of antibody to CTLA4. In collaboration with Dr. James P. Allison of Memorial Sloan Kettering, we would inject 9H10, a hamster anti-mouse anti-CTLA4 antibody to blunt the immunosuppressive signals generated in T cells that infiltrate HT treated tumors. In addition, we will examine whether anti-CTLA4 antibody administration can help us break the tolerance to heat activated tumor antigens.

B. KEY RESEARCH ACCOMPLISHMENTS:

- HT induces apoptosis in RM1 cells both in vitro and in vivo.
- DCs engulf heat treated apoptotic RM1 tumor cells.
- Tumor antigens released from HT-treated RM1 cells provide activation signals to intratumoral DCs, resulting in elevated expression of cell surface T-cell-costimulatory molecules (CD86 and CD80).
- Intratumoral inoculation of DC following heat treatment significantly increases tumoricidal effects of HT. It further induces a tumor specific T helper and cytotoxic lymphocyte response.
- Administration of systemic AdGM-CSF increases the intratumoral infiltration of DC, CD4+ and CD8+ T lymphocytes and delays RM-1 tumor recurrence after hyperthermia.
- Tumor growth inhibition and induction of T cell mediated immunity was maximally enhanced by a combination of GMCSF, intratumoral DC, and HT.

D. REPORTABLE OUTCOMES:

1) AACR presentation, 2005.
2) Manuscript under preparartion. Localized tumor hyperthermia combined with intratumoral dendritic cell injection induces systemic antitumor immunity.
3) Development of a metastatic RM-1 subclone, RM-1Lu.

E. CONCLUSIONS:

DC-based therapy, such as, intratumoral DC injection or systemic administration of GM-CSF, might augment the tumoricidal effects of local tumor hyperthermia in recurrent prostate cancer. Furthermore, immunotherapy with DC or GM-CSF induces a tumor-specific immune response that activates both helper and cytotoxic T cells. Experiments are underway to prolong and amplify the HT-induced immunity by blocking T cell inhbitory receptors, CTLA4 and inducing DC maturation with soluble CD40L and ATRA.

F. REFERENCES:

1) Arunika Mukhopadhaya, Joseph Mendecki, Kosho Yamanouchi, Laibin Liu, Shalin Shah, Indranil Basu, Alan Alfieri, Madhur Garg, Shalom Kalnicki, and Chandan Guha. Localized hyperthermia combined with intratumoral dendritic cell injection induces systemic antitumor immunity. Proc Amer Assoc Cancer Res 2005;46:725.

G. APPENDICES:

AACR abstract 2005: Proc Amer Assoc Cancer Res 2005;46:725.

725 Localized hyperthermia combined with intratumoral dendritic cell injection induces systemic antitumor immunity

Arunika Mukhopadhaya, Joseph Mendecki, Kosho Yamanouchi, Laibin Liu, Shalin Shah, Indranil Basu, Alan Alfieri, Madhur Garg, Shalom Kalnicki, Chandan Guha. Albert Einstein College of Medicine, Bronx, NY and Montefiore Medical Center, Albert Einstein College of Medicine, Bronx, NY.

Prostate adenocarcinoma when treated with localized tumor hyperthermia (LTH) can potentially serve as a source of tumor antigen, where dying apoptotic/necrotic cells release tumor peptides slowly over time. In addition, LTH treated cells can release heat shock proteins that can chaperone antigenic peptides to antigen-presenting cells (APCs), such as dendritic cells (DCs). We attempted to discern whether sequential application of LTH and intratumoral DC inoculation would activate antitumor immune response in a syngeneic murine model of prostate cancer (RM-1). Three-week old RM1 (approx. 100mg) tumors, grown in the dorsum of foot of C57BL/6 male mice, were subjected to LTH (43.7°C for 1 hr) x 2, separated by 5days. Following second LTH treatment, animals received either PBS or DCs (106 /injection) intratumorally every three days for three injections. Separate cohorts also received systemic murine GM-CSF, via injection of recombinant adenovirus (8x109 particles), 1 day after LTH. Control animals received adenoLacZ or adenoGFP. RM1 tumors were followed by volume measurement. Antitumor immune response was measured by cytokine release assays, ELISPOT and LDH release to measure T helper and CTL response of splenocytes. LTH alone (n=10) was responsible for tumor growth delay when compared to untreated controls. Intratumoral DC inoculation (n=16) augmented the tumoricidal effects of LTH, significantly (p=0.007). Systemic injection of control adenoviruses (LacZ and GFP) did not alter tumor control of LTH-treated animals (p=0.67). In contrast, administration of AdGMCSF (n=12) further enhanced the tumoricidal effects of LTH+DC (p<0.001). Intratumoral DC injection induced tumor-specific T-helper cell activity (IFN□ ELISPOTS) and CTL activity (LDH release 17-20% vs 5%). Ad-GM-CSF augmented the CTL response to 36.3% (range, 30-44%), indicating amplification of tumor-specific immune response by adding GM-CSF as an adjuvant. The combination of LTH, AdGMCSF and intratumor DC inoculation induced a strong systemic tumor-specific immune response and enhanced local control of RM-1 tumors. Immunomodulation of LTH could, therefore, be useful in the treatment of local and systemic recurrence of prostate cancer.

Citation format: Proc Amer Assoc Cancer Res 2005;46:725.